

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
4 September 2003 (04.09.2003)

PCT

(10) International Publication Number  
**WO 03/072613 A1**

- (51) International Patent Classification<sup>7</sup>: **C08B 37/10**, A61L 33/08 (74) Agent: **BARCIELLI, Giovanna**; Nicox Research Institute S.r.l, Via Ariosto, 21, 20091 Bresso- MI (IT).
- (21) International Application Number: PCT/EP03/02093 (81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.
- (22) International Filing Date: 28 February 2003 (28.02.2003)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data: 02290492.4 28 February 2002 (28.02.2002) EP
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- (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Declaration under Rule 4.17:**

— *of inventorship (Rule 4.17(iv)) for US only*

**Published:**

— *with international search report*

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

WO 03/072613 A1

(54) Title: NITRO-DERIVATIVES OF EPOXYHEPARIN

(57) Abstract: The present invention relates to heparin, heparin fragments and heparin mimetics, functionalized by introduction of nitro groups and formation of epoxy groups. It also relates to the process for their preparation comprising the following steps: a. introduction of an epoxy functionality on positions 2,3 of the uronic acid; b. nitration of the epoxyheparin obtained in step a.

### Nitro-derivatives of Epoxyheparin

#### Technical Field

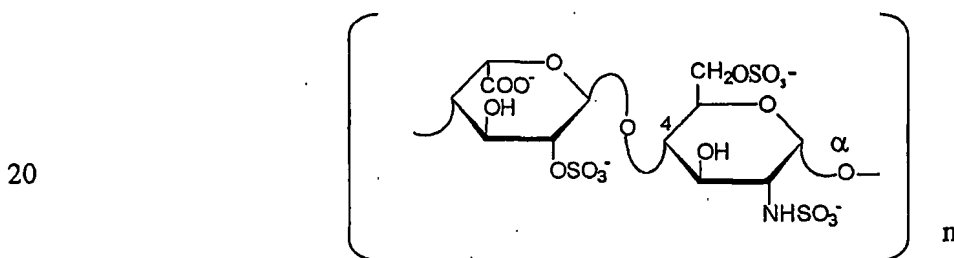
The present invention relates to new functionalized heparin for use in the preparation of materials presenting an anti-proliferating activity in surgical devices.

The compounds according to the invention are derivatives of heparin functionalized by formation of epoxide groups and nitro groups.

#### 10 Background Art

It is known the anti-thrombotic activity of heparin, that makes it suitable to come in contact with biological fluids or tissues. Heparin is constituted by disaccharidic repetitive unities containing a residue of uronic acid and a residue of hexosamine.

The prevalent repetitive unities of heparin are represented in scheme 1 wherein n usually varies from 8 to 35.



Schema 1: prevailing structure of heparin

25 It is known the use of heparin as agent to be supported on the surface of polymer materials in order to improve their compatibility with organic tissues or fluids. For example in US 4,987,181 depolymerized heparin is covalently bonded to an olefin copolymer. The resulting product is then used as adhesive film to be applied on polymeric surfaces that must come in contact with organic fluids or tissues. In  
30 such process the depolymerization is useful to create the functional group necessary to the formation of the covalent bond, but it also gives rise to the

decrease of some biological activities, such as the anti coagulant activity. In US 5,541,305 it is obtained a material compatible with blood by heparinizing with alkali salts of heparin sulphate groups a polymer having quaternary amino groups. WO 99/27976 discloses a polymer material with high compatibility with organic fluids and tissues comprising a basic polymer which is covalently bonded to to molecules of glycosaminoglycans. The glycosaminoglycans are bonded to the polymer through functionalization by formation of amino or epoxy groups.

The functionalization of polysaccharides with groups capable of releasing nitric oxide has been proposed in US 6,261,594. It discloses a nitric oxide donor comprising a modified chitosan polymer and a nitric oxide dimer ( $N_2O_2$ ) directly bonded to the polymer backbone.

The object of the present invention is the provision of new functionalized heparin comprising epoxy functionalities and nitro functionalities. These compounds are easily fixed onto surfaces comprising functional groups and make them not only highly compatible with organic fluids or tissues, but also active against cell proliferation that takes place after surgical insertion of the same in the human body.

Another object of the present invention is the provision of a simple process for the production of said materials.

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#### **Disclosure of the Invention**


The compounds object of the present invention having anti-inflammatory, non thrombogenic surfaces with an enhanced compatibility with organic fluids and tissues, are heparin, heparin fragments or heparin mimetics functionalized by introduction of nitro groups and formation of epoxy groups.

The epoxy group can be used to covalently bond heparin to a substrate which contains functional groups (e.g. hydroxyl groups).

The covalent bond is preferably formed through an amination reaction of the epoxy groups obtained on the uronic acid residues with ammonia or primary amines; more preferably the amination reaction is made with ammonia.

30

In alternative, the polysaccharide can be covalently bonded to the basic polymer through the use of spacers of general formula  $T-R^{II}-T$  wherein:  $R^{II}$  is selected from the group comprising: linear or branched  $C_1-C_{20}$  alkylene,  $C_4-C_{20}$  cycloalkylene,  $C_6-C_{20}$  arylene,  $C_7-C_{20}$  arylalkylene; T is selected from the group

- 5 comprising:  $NR^I_2$ ,  $Si(OR^I)_3$ ,  $COR^{III}$ ,  $OR^I$ , X,  wherein  $R^{III}$  is selected from the group comprising: Cl, Br, I.

T can also represent a group from which through simple reactions it is possible to obtain one of the groups described, in case the compound  $T-R^{II}-T$  does not prove to be stable. For example compounds of formula  $NH_2-(CH_2)_nCHO$  wherein n  
10 varies from 2 to 20 do not prove to be stable as they would tend to polymerize, it is possible anyway to use compounds of formula  $NH_2-(CH_2)_nCH(OEt)_2$  wherein the aldehydic group is present as an acetal and can be restored through hydrolysis. Examples of groups  $T-R^{II}-T$  are: diaminobutane, diaminopentane, diaminohexane, diaminoheptane, 1,4(aminoethyl)benzene, 1,4 diaminobenzene, 1-amino-4-  
15 trimetoxysilylbutane, 1-amino-4-trimetoxysilylpentane, 4-methyl(trimetoxysilyl)-benzylamine, ethylenglycol, 1,6-dihydroxyhexane, 1,4-dibromomethylbenzene.

Preferred spacers are compounds of general formula  $NH_2-(CH_2)_n-NH_2$  wherein n varies from 1 to 12, more preferably from 3 to 8.

The process for the preparation of compounds according to the invention  
20 comprises the following steps:

- a. introduction of an epoxy functionality on positions 2,3 of the uronic acid;
- b. nitration of the epoxyheparin obtained in step a)

Though the temperature in step a) is not critical, it preferably varies between 10°C  
25 and 80°C, more preferably between 40°C and 70°C; the reaction time preferably varies between 10 minutes and 3 days.

In the epoxidation reaction, the quantity of groups that reacted in comparison with the total quantity of reactive groups is indicated as a percentage. The used functionalization percentage influences whether and how heparin is bonded to the  
30 basic polymer, but it also influences the biological activity of the obtained

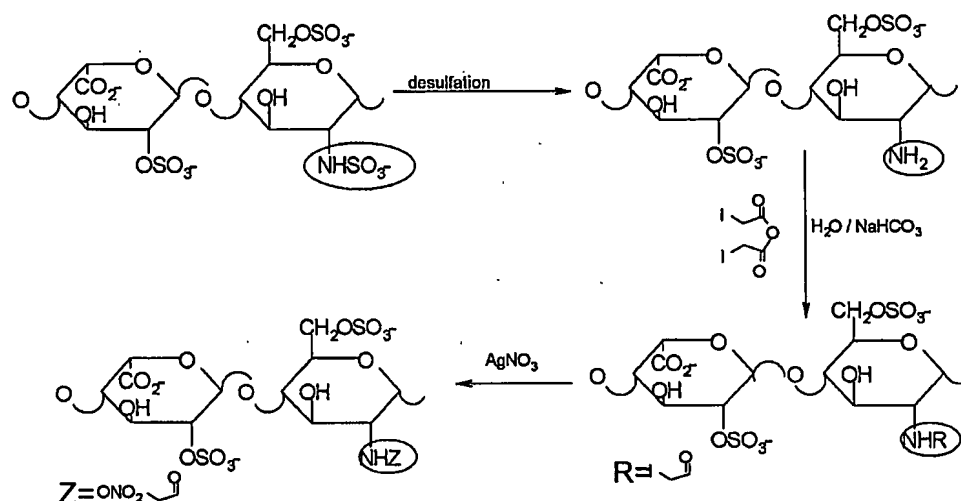
polymer material. The preferred percentage of groups that reacts in the epoxidation reactions is comprised between 5 and 60%, more preferably 10-40%. The nitro group can be covalently bonded to the saccharide structure either directly (e.g. through nitration of a carbon of the saccharide unit) or through a  
5 divalent radical acting as a spacer between the saccharide unit and the nitro group. Suitable spacers are divalent radicals derived from C<sub>2</sub>-C<sub>20</sub> aliphatic or aromatic hydrocarbons, ethers, polyethers, carboxylic acids or their derivatives, and the like.

A first path to the preparation of nitro-epoxyheparin according to the invention is  
10 by direct nitration by means of a nitrating mixture. The reaction is preferably carried out by first preparing the nitrating mixture, wherein the previously dried epoxy-heparin is added in small amounts under stirring. Examples of nitrating mixtures are sulfuric acid-nitric acid, phosphoric acid-nitric acid, acetic anhydride-nitric acid, nitrous oxide-sulfuric acid. Preferred mixtures are the  
15 mixture sulfuric acid-nitric acid, also called sulfo-nitric mixture and the mixture acetic anhydride-nitric acid. More preferred mixture is the mixture acetic anhydride-nitric acid.

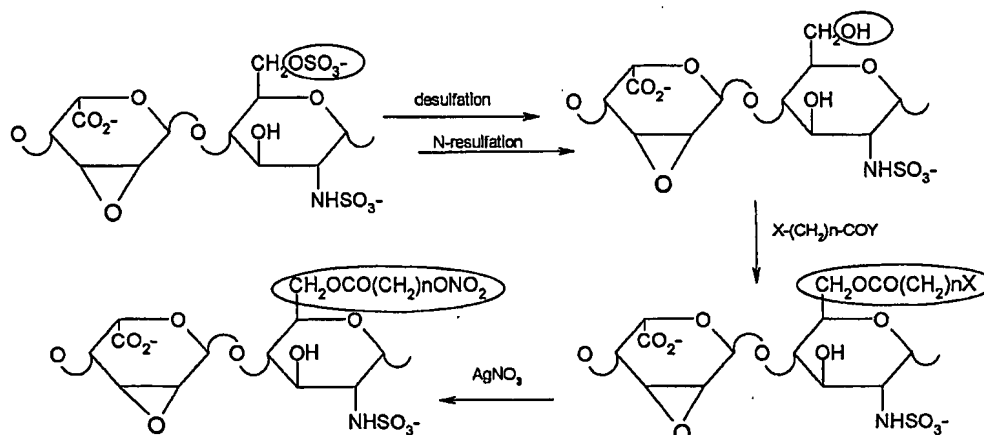
Using the latter mixture, the molar ratio between acetic anhydride and nitric acid can vary in a broad range and is preferably comprised between 1:1 and 1:2, more  
20 preferably between 1:1.4 and 1:1.6. The amount of nitro groups per saccharide unit also varies considerably, and the ratio in equivalents between saccharide units and nitro groups is preferably comprised between 0.2 and 1.0, more preferably comprised between 0.3 and 0.8.

Another synthetic path for the synthesis of nitro-epoxyheparin according to the  
25 invention is by haloacylation followed by nitration. In this case, it is possible to prepare first epoxy-heparin containing N-desulfated hexosamine. The N-desulfated hexosamine is then reacted with a halo anhydride such as iodoacetic anhydride, obtaining the corresponding amide in position 2 of the hexosamine. The iodine atom is then substituted with a nitro group by reaction with a suitable  
30 nitrate. By using the suitable reaction conditions, it is possible to dose the amount of N-desulfation and, consequently, the amount of nitro groups introduced.

Examples of suitable nitrates are  $\text{AgNO}_3$  and  $\text{NBu}_4\text{NO}_3$ . It is possible to use anhydrides different from iodoacetic anhydride, such as the anhydride of a  $\omega$ -halocarboxylic acid, obtaining in this way nitro-epoxyheparin having a different spacer group. Preferred halogens are iodine and bromine. More preferred is iodine. Using heparin fragments and iodoacetic anhydride as starting materials, the reaction scheme for a non epoxidized disaccharide unit is the following:

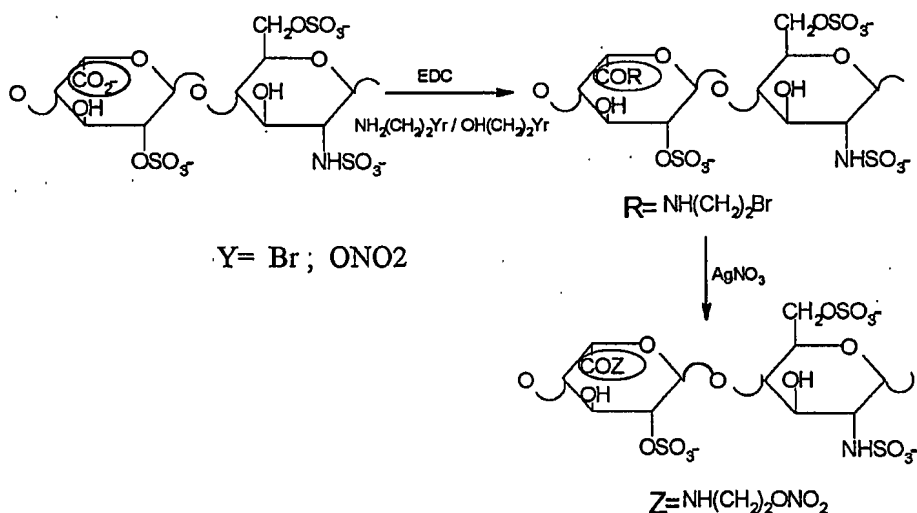


It is also possible to acylate the position 6 of desulfated hexosamine. In this case it is preferable to use compounds of formula  $\text{X}-(\text{CH}_2)_n-\text{COY}$  wherein X is chlorine, bromine, iodine or tosyl; preferably X is bromine; Y is chlorine, mercaptothiazole, mercaptobenzothiazole, mercaptobenzoxazole, p-nitrophenol. The reaction scheme for an epoxidized disaccharide unit is the following:



Another possible route of preparation of nitroderivatives is described in the following scheme, where the carboxyl group is activated by reaction with di cyclohexylcarbodiimide and then reacted with a compound of general formula  $X(CH_2)_nY$  where Y is Cl, Br, I, O-tosyl,  $ONO_2$  and X is OH or  $NH_2$ :

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The nitro groups are measured by using the ratio functional group/saccharide, i.e. it is expressed in terms of nitro groups per saccharide ring. This ratio influences the biological activity of the obtained heparin. Depending on the final use of the compounds, it is possible to use high MW heparin ( $10,000 < Mw < 18,000$ ), low MW heparin ( $3,000 < Mw < 10,000$ ) or very low MW heparin ( $Mw < 3,000$ ). The latter also includes heparin mimetics, i.e. synthetic oligosaccharides which reproduce sequences present in natural heparin (see for example EP 84999 and EP 300099).

The present invention is now more deeply described through examples that must be considered explanatory but that are not intended to limit in any way the invention itself.

20

### **Experimental section**

The reactions have been carried out on the following substrates:

5      1. epoxyheparin 30%

5 g of heparin is dissolved in 63 ml of a solution NaOH 1 N. The solution is left under stirring stir for 45 min at 60°C, cooled and neutralized with diluted HCl. Then, the solution was dialyzed dialysis tubes with a 2000Da cut-off against water for 78 hours.

10      % epoxy group: 30% by  $^{13}\text{C}$  NMR

2. epoxyheparin 24%

1 g of heparin is dissolved in 12 ml of a solution NaOH 1 N. The solution is left under stirring for 45 min at 60°C, cooled and neutralized with diluted HCl. Then, the solution was dialyzed dialysis tubes with a 2000Da cut-off against water for 78 hours.

15      % epoxy group: 24%  $^{13}\text{C}$  NMR

#### **Example 1**

##### **Preparation of nitro-epoxyheparin via nitration of epoxy-heparin 30% with nitro-acetic mixture**

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Nitroacetic mixture was prepared by dropping, over 45 minutes, 20.8 ml of cold acetic anhydride in 31.2 ml of nitric acid (90%) kept under stirring and cooled at -20°C in an  $\text{N}_2$ /acetone bath. At the end, 130 mg of HEP were added. When the temperature was stabilized,  $\text{N}_2$ /acetone bath was changed with an ice bath; the mixture was stirred 4 hours at 0° C. The mixture was poured into 100 ml of a mixture diethyl ether – n-hexane 1:1 at -40°C in an  $\text{N}_2$ /acetone bath. After 1 hour the sample is filtered, the precipitate dissolved in water, neutralized with a solution of diluted NaOH. The sample is dialyzed in dialysis tubes with a 2,000 Da cut-off against water for 78 hours. At the end, the sample is concentrated through evaporation at a reduced pressure and at a low temperature. The product is purified by gel filtration on Sephadex G25.

30



The product has the following characteristics:

Ethero correlate NMR spectra presence of a signal at : 83 ppm; 5,5 ppm. The amount of epoxy groups is still 23%.

Elemental analysis: C: 22.51 %; N: 5.71 %;

5                                      N per saccharide: 1.3  
  ONO<sub>2</sub> per saccharide: 0.8

Yield = 72.5%

#### Example 2

#### 10    Derivatization of nitro-epoxy-heparins with Diaminobutane.

To a 4% solution of nitro-epoxy-heparin of example 1 (50 mg in 1.25 ml of water) 12.5 µl of DAB is added and it is left for 50 hours at RT. The solution is precipitated with EtOH (4v/v), after adding 37 mg of NaCl in order to ease the precipitation. The sample is dialyzed in dialysis tubes with a 1000Da cut-off  
15    against water for 48 hours. At the end, the sample is concentrated through evaporation at a reduced pressure and at a low temperature.

### Claims

1. Polysaccharide characterized in that it is constituted of heparin, heparin  
5 fragments and heparin mimetics, functionalized by introduction of nitro  
groups and formation of epoxy groups.
2. Polysaccharides according to claim 1 wherein the percentage of  
epoxidation is comprised between 5 and 60%.
3. Polysaccharide according to claims 1-2 wherein the ratio in equivalents  
10 between saccharide units and nitro groups is comprised between 0.2 and  
1.0.
4. Polysaccharides according to claims 1-3 wherein the polysaccharide is  
heparin having Mw comprised between 10,000 and 18,000 Da.
5. Polysaccharides according to claims 1-3 wherein the polysaccharide is an  
15 heparin fragment having Mw comprised between 3,000 and 10,000 Da.
6. Polysaccharides according to claims 1-3 wherein the polysaccharide is an  
heparin fragment having Mw lower than 3,000 Da.
7. Polysaccharides according to claims 1-3 wherein the polysaccharide is an  
heparin mimetic
- 20 8. Process for the preparation of polysaccharides according to claim 1  
comprising the following steps:
  - a. introduction of an epoxy functionality on positions 2,3 of the  
uronic acid;
  - b. nitration of the epoxyheparin obtained in step a.
- 25 9. Process according to claim 4 wherein step b. is performed using a mixture  
acetic anhydride-nitric acid.
10. Process according to claim 5, wherein the molar ratio between acetic  
anhydride and nitric acid is comprised between 1:1 and 1:2.

## INTERNATIONAL SEARCH REPORT

Intern: .pplication No  
PCT/EP J3/02093

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 7 C08B37/10 A61L33/08

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 7 C08B A61L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

WPI Data, PAJ

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages                              | Relevant to claim No. |
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☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the International search

3 June 2003

Date of mailing of the International search report

13/06/2003

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## INTERNATIONAL SEARCH REPORT

 Intern:      plication No  
 PCT/EP 03/02093

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